Are biochemical biomarker responses related to physiological performance of juvenile sea bass (Dicentrarchus labrax) and turbot (Scophthalmus maximus) caged in a polluted harbour ?

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Abstract

Biomarker responses to toxic exposure have been used for decades to indicate stress in aquatic organisms, or the magnitude of environmental pollution. However, little has been done to compare the simultaneous responses of both biochemical and physiological biomarkers. The purpose of this study was twofold. Firstly to analyse the responses of several biochemical biomarkers measured on juvenile sea bass and turbot caged in a northern France harbour at a reference and contaminated stations. Several biotransformation parameters (Ethoxyresorufin-O-deethylase –EROD– and Glutathione S-transferase –GST) and an antioxidant enzyme (Catalase –CAT) were analysed. Secondly, to compare their responses to several growth and condition indices, measured on the same fish. In the contaminated station, EROD and GST activities were found to be significantly higher, and a decrease of CAT activity was observed for both species. For individual sea bass, biochemical biomarkers showed numerous significant correlations with growth and condition indices, such as the Fulton's K condition index, the RNA:DNA ratio and the lipid storage index. On the contrary, there were only a few significant correlations for turbot, suggesting a species-specific response. Our study indicates that the analysis of the simultaneous responses of both biochemical and physiological biomarkers can be useful for monitoring complex exposure and to assess habitat quality.

Keywords : biochemical biomarker, fish growth, condition indices, metals, PAHs, caging

1. Introduction

A multitude of xenobiotics contaminate the marine environment, and although chemical analyses are able to measure many of these compounds qualitatively and quantitatively, complex mixtures of these chemical pollutants cannot be fully assessed. Furthermore, chemical analyses alone do not reveal the impact of chemical pollution on the aquatic environment because of potential synergistic/antagonistic effects of complex mixtures of chemical pollutants. In this context, utilisation of biomarkers as an early warning of pollution or degradation in ecosystems has increased over the past 20 years (Adams, 2002).

Identifying a suitable biomarker of toxicity represents a major challenge in current ecotoxicological research (Cheung et al., 2007). Many parameters have been investigated to assess disturbances of various physiological functions linked to chemical exposure (Van der Oost et al., 2003). Peakall (1994) defined biomarkers as "biological responses than can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals". The subdivision of biomarkers in literature is rather diffuse since the impact of toxic xenobiotics on fish has been analysed with various types of exposure and effect biomarkers, ranging from molecular, through cellular and physiological responses, to behavioural changes. In order to avoid confusion, in this paper, biomarkers acting at the subcellular level as biotransformation process or oxidative stress protection will be referred to as "biochemical biomarkers" and biomarkers reflecting fish growth or physiological condition will be referred to as "physiological biomarkers".

Because many toxic effects initially occur at the subcellular level, there has been an increasing use of biochemical biomarkers that help to determine causative agents responsible for altered cellular function (Schlenk et al., 1996). Among the biochemical biomarkers described in relating literature, phase I and phase II biotransformation parameters such as EROD (Ethoxyresorufin-O-deethylase) and GST (Glutathion-S-transferase) activities are currently used in environmental risk assessment (Sanchez et al., 2008). Biotransformation of chemicals is a requisite for detoxification and excretion (Gravato and Santos, 2003). The first step is usually catalysed by cytochrome P450-dependent monooxygenases (phase I) and their products, or several others organic pollutants, are subsequently coupled to endogenous metabolites (phase II) (Buhler and Williams, 1988; Landis and Yu, 1995). Antioxidant enzymes are also commonly used to understand the associated toxic-mechanisms of xenobiotics (Sanchez et al., 2005; Oliveira et al., 2008). Many pollutants exert their effects through redoxcycling, resulting in the production of reactive oxygen species (ROS). The role

of antioxidant systems is to protect the cells from this oxidative stress. Thus, measurement of components of the antioxidant defence system may be helpful to determine organism exposure to pollutant (Bilbao et al., 2010).

Attempting to relate biomarker responses of individual organisms to increasing pollutant exposure and stress, both in the laboratory and *in situ,* offers considerable potential for improving the ecological relevance of ecotoxicological test procedures (Depledge et al., 1995). However, although the role of biochemical biomarkers as early warning tools is recognised, it is difficult to understand their significance at higher levels of biological organisation. Indeed, in spite of their rapid responsiveness and sensitivity to contaminant exposure, biochemical biomarkers have questionable ecological relevance, as a result of being endpoints at a low level of biological organisation (Castro, 2004). On the contrary, changes in physiology and fitness seem to be a common response in marine organism exposed to stressful pollutants (Alquezar et al., 2006; Faucher et al., 2008). Pollutants can induce various biological responses in fish, affecting the organisms from the biochemical to the population-community levels (Adams, 2002). For example, many investigations on biological responses of fish populations to contamination indicated a general decrease of the relative fecundity, the growth rate and condition factor in contaminated estuaries (Laroche et al., 2002; Marchand et al., 2003; Amara et al., 2007). It is likely that changes in individual health manifest themselves at higher levels of ecological organisation, leading to reduced fish recruitment, abundance and production.

It has long been suggested that biochemical biomarkers should be used in conjunction with measurements of fitness (Anderson et al., 1994; Depledge et al., 1995). However, few studies have demonstrated correlative relationships between biochemical biomarkers responses and reduced fitness of aquatic organisms exposed to toxicants (Depledge et al., 1995; Lesser et al., 2001; Fonseca et al., 2009). Biological impairment (e.g. embryonic malformations, growth and condition depletion, fecundity and pollution tolerance) may directly affect the survivorship of organisms. Therefore, this type of multifaceted approach is important because it will improve our ability to use molecular biomarker responses of organisms to predict higher-level consequences of toxicant exposure (Rose et al., 2006).

Although biochemical or physiological biomarkers are intended to be useful tools for environmental assessment in the field, most of them have been developed under laboratory controlled toxic conditions. These studies often fail to recreate the mixed exposure situations occurring in nature. However, in field situations, the migration of many fish species for feeding and breeding creates uncertainty about how individuals sampled in some habitat truly

reflects the water quality around the site of capture. In comparison, a caging strategy may, in specific situations, give more realistic results in studies of bioavailability, bioaccumulation and biological effects of contaminants in fish. The technique of caging offers advantages over organism chemical exposure (Oikari, 2006) with a control of the precise location and duration of exposure while environmental field conditions are preserved. The use of cage-held animals from a common source (e.g. hatchery) also removes the potential for genotype adaptation, which is a distinct possibility in feral fish populations exposed to contaminants (Winter et al., 2005). The development of caging field experiments would provide an indication of the impact of contamination on marine fish and information on the applicability of this experimental strategy for assessing habitat quality.

The present study was designed to analyse the responses of two biotransformation parameters (EROD and GST activities) and an antioxidant enzyme (catalase) on the basis of a caging field experiment of two marine fish species in a polluted area (Boulogne sur Mer). In a second part, the responses of these enzymatic biomarkers were compared to different growth rates (in length and weight) and condition indices, measured on the same individual fish and analysed in a previous study (Kerambrun et al., submitted for publication). We used three condition indices: the Fulton's K condition factor; the RNA:DNA ratio which is used in numerous studies as indices for nutritional condition and growth assessment in larvae and juvenile fish (Buckley, 1984; Gwack and Tanaka, 2001; Amara et al., 2009); and a lipid storage index based on the ratio of the quantity of triacylglycerols (TAG; reserve lipids) to the quantity of sterols (ST; structural lipids) in fish (Amara et al., 2007).

2. Material and Methods

This experiment was conducted in accordance with the Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. The University of Littoral Côte d'Opale is authorised to conduct experimentation on animals in its capacity as a certified establishment; according to the administrative order N° B62-160-2.

2.1. Experimental caging technique

Cage placement was carried out in June 2009, in an important northern France harbour (Boulogne sur Mer) (Fig. 1). This harbour is impacted by municipal and industrial discharges,

fishing and shipping activities, and marinas. Two locations were chosen: station A, in front of the harbour (depth 5 - 10 m according to tide) and station B, in the inner part of the harbour (depth 4 m). Previous data have shown that sediments from station A contain relatively low levels of metals and PAHs whereas those from station B are significantly more contaminated with metals and PAHs. The distance between the two stations was about 2.5 km. 48 juvenile sea bass (*Dicentrarchus labrax*), (Weight: 2.84 ± 0.79 g; Age: 4 months) and 48 juvenile turbot (*Scophthalmus maximus*), (Weight: 4.62 ± 0.72 g; Age: 3 months) were obtained from hatcheries (Aquanord and France Turbot, in France). Before the caging procedure, the fish were acclimatised for 2 weeks in a clean tank (2.7 m^3), supplied with an open seawater circuit. The water was aerated with air pumps, and the photoperiod was set at a 10 h light and 14 h dark cycle. During the acclimation, water temperature was about 13 ± 1 °C and the fish were progressively fed with artemia and pieces of shrimps and mussels to acclimate them to natural prey consumption. One day before the caging experiment, they were anaesthetised (2-phenoxyethanol), weighed (near to 0.01 g), measured for total length (near to 0.1 mm) and individually marked (Visual Implant Tag, 1.2 mm x 2.7 mm, Northwest Marine Technology). For each species, fifteen fish were sampled before the beginning of the experiment to establish t_0 values, used as reference. Cylindrical cages (0.7 m height, 0.65 m diameter, 230 L volume), made from polypropylene mesh (10 mm) were used for the sea bass. Rectangular cages (0.3 m height, 0.6 m width, 0.9 length, 160 L volume) were chosen for the turbot, benthic fish, in order to increase the surface area in contact with the sediment. At each station, the two types of cage were immersed with fish randomly placed and then fixed to the bottom with a screw anchor by scuba-divers. Precisely, 15 sea bass and 17 turbot were placed in station A, and 18 sea bass and 16 turbot, in station B (initially, 18 fish of each species were provided in each cage, but some fish escaped when they were put in the cages due to bad weather conditions). Sea water physicochemical parameters (temperature, salinity, oxygen and pH) were measured, and bottom sediment sampled for chemical contaminant analysis. Following the 38−days exposure, all the fish were rapidly transferred to the laboratory (within 2 h), anaesthetised (2−phenoxyethanol), identified (tag), weighed and measured for total length. Livers were sampled, frozen in liquid nitrogen and stored at −80 °C. Muscles were sampled and preserved at –20 °C and otoliths (sagittae) extracted and preserved in ethanol (95%).

2.2. Sediment analysis

Sediment samples were collected near to each cage location using a Van Veen grab (250 cm^2 sampling area) in order to determine metals, PAHs and PCBs contents.

In order to determine selected metals (Cd, Cr, Cu, Ni, Pb, and Zn) in the total fractions, sediments were dried at 40 °C to constant weight and were ground into a powder. About 0.250 g ground sediments were digested with HF (Suprapur, Merck) at 110 °C for 48 h followed by a mixture of concentrated acids HCl:HNO_3 (3:1, v:v, Suprapur Merck) at 120 °C for 24 h, operation renewed once. Each heavy metal was measured by inductively coupled plasma−atomic emission spectrometry (ICP−AES, VARIAN Vista Pro, axial view).

Total Hg was measured in dry and ground sediment samples (without any pre−treatment) by means of atomic absorption spectroscopy (AAS) using an AMA 254 solid phase Hg−Analyser (Altec Ltd., Prague, Czech Republic) (Ouddane et al., 2008).

The persistent organic pollutants, including PAHs (EPA's 16 priority PAHs) and PCBs (7 congeners) were analysed. In brief, organic compounds were extracted from 2 g of dried sediment by microwave (120 °C for 15 min, 1200 W) assisted extraction with 40 mL of a mixture of acetone and hexane (1:1, v:v). Simultaneous determination of PAHs and PCBs was performed on a gas chromatography−mass spectrometry (GC−MS, VARIAN, CP 3800 – 1200 MS TQ). A ZB−MultiResidue column (30 m, 0.25 mm, 0.25 µm) were used (Phenomenex). Identification of PAH compounds and PCB congeners was based on the comparison of their GC−retention times and their mass spectrum, with appropriate individual standards.

2.3. Biochemical biomarkers analysis

Livers were homogenised in an ice-cold phosphate buffer (0.1 M, pH 7.8) with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor. The homogenates were centrifuged at 10,000 g at 4 °C, for 15 min and the post-mitochondrial fractions were used for biochemical assays. Total protein concentrations were determined using the method of Bradford (1976) with bovine serum albumin (Sigma-Aldrich Chemicals, France) as a standard.

Ethoxyresorufin-O-deethylase activity (EROD) was determined following the hydroxylation of 7-ethoxyresorufin by the method of Flammarion et al. (1998). The reaction mixture consisted of a phosphate buffer (0.1 M, pH 6.5), 7-ethoxyresorufin (8 µM) and NADPH (0.5 mM). The change in fluorescence was recorded (excitation wavelength 530 nm,

emission wavelength 585 nm) and enzyme activity calculated as pmol.min^{-1}.mg^{-1} protein using a Resorufin standard.

Glutathione S-transferase activity (GST) was determined following the conjugation of reduced glutathione with CDNB by the method of Habig et al. (1974). The reaction mixture consisted of a phosphate buffer (0.1 M, pH 6.5), reduced glutathione (1 mM) and CDNB (1 mM). The change in absorbance was recorded at 340 nm and enzyme activity calculated as µmol.min^{-1}.mg^{-1} protein using GST standard.

Catalase activity (CAT) activity was determined by the method of Babo and Vasseur (1992). In brief, the assay mixture consisted of a phosphate buffer (100 mM pH 6.5) and H_2O_2 (28 mM). Change in absorbance was recorded at 240 nm. CAT activity was calculated in terms of µmol.min^{-1}.mg^{-1} protein, using bovine erythrocyte Catalase as standard.

2.4. Physiological parameters

2.4.1. Growth index

Sea bass and turbot specific growth rates in weight (% per day) were estimated as :

$$GW = 100(\ln W_2 - \ln W_1)/(t_2 - t_1),$$

where (W_1) and (W_2) are fish total body weight at times (t_1) (beginning of the experiment) and t_2 (time of collection). Similarly, the specific growth rate in length was estimated as :

$$GL = 100(\ln L_2 - \ln L_1)/(t_2 - t_1),$$

where (L_1) and (L_2) are fish total length at times (t_1) and (t_2) respectively.

The recent growth index (RG) was determined by measuring the width of the peripheral daily increments of the otoliths from the previous 5 days before the end of the experiment. Sagittae were removed, cleaned and mounted on a glass side in thermoplastic cement (Crystal Bond). Sections of sagittae were obtained by polishing them in both side with grinding paper of decreasing grit sizes (5 to 0.1 µm) until increments at the outer edge were visible. Otoliths were etched for 30 sec with 0.1 M EDTA and analysed under transmitted light, using a video system fitted to a compound microscope (TNPC 5.0, NOESIS) (Amara et al., 2009).

2.4.2. Condition Indices

We estimated three condition indices for the fish caged in the two stations and in the fish sacrificed at the beginning of the experiment (t_0): RNA:DNA ratio and TAG:ST ratio as indicators of nutritional status and Fulton's K condition index as an indicator of the fishes general well being. We calculated Fulton's K condition index with the formula :

$$K = 100(W/L^3),$$

where (W) is the body mass (mg) and (L) is the total length (mm).

The procedure used to determine RNA and DNA concentrations in individual fish was based on the Clemmesen method (1988), slightly modified as described by Amara et al. (2009). The quantity of RNA and DNA was determined by the fluorescence−photometric technique using a specific nucleic acid fluorescent dye−ethidium bromide.

The amount of total lipids in each individual was measured on a sample of lyophilised muscle (0.07 g). Lipid extraction was conducted using the method of Bligh and Dyer (1959), slightly modified as described by Amara et al. (2007). TAGs and sterols were separated from other lipids by performing thin layer chromatography (TLC).

2.5. Statistical analysis

Statistics were performed with Xlstat 2007. Mean comparisons of biomarker responses between t_0 and fish caged in station A and B were analysed using one-way ANOVA, followed by post-hoc Tukey tests. As biochemical and physiological biomarkers were analysed on the same fish, a Pearson product moment correlation matrix was computed using data from all the individuals collected from both stations, to examine the statistic links between parameters. A principal component analysis (PCA) was used to evaluate the relationships between the chemical sediment contaminants and fish biological responses.

3. Results

3.1. Environmental parameters

At the beginning of the experiment, bottom temperature (station A : 14.6°C ; station B : 14.9°C), salinity (station A : 34.1 PSU; station B : 33.5 PSU) and oxygen contents (station A : 7.86 $mg.L^{-1}$; station B : 7.04 $mg.L^{-1}$) were similar in the two caging stations. The studied area temperature had increased during June and July; the temperature was 18°C in both stations at the end of the caging exposure, which was also the highest temperature during the caging experiment.

The results of metal concentrations expressed as mg. kg^{-1} of dry sediment are reported for each station in Fig. 2, PAHs and PCBs concentrations in Table 1. Sediment samples indicated different levels of contamination in metallic and organic compounds. As expected, all metal concentrations analysed in sediment collected from station A, located in front of the harbour, were lower than station B and no PAHs were detected. A way to assess sediment toxicity is the use of contamination sediment quality guidelines. Long et al. (1995) identified two guideline values: the effects range−low (ERL) and the effects range−median (ERM). Concentrations above the ERL, represent a possible−effects range within effects that would occasionally occur. The concentrations above the ERM values represent a probable−effects range within which effects would frequently occur. In our study, all chemical contaminants detected in station A were found to be lower than the toxic effect range ERL. Sediment from station B exhibited concentrations in Cd, Hg and Zn higher than ERL and lower than ERM. The PAHs concentrations stayed below the guideline values and no PCBs was detected at any station (< 0.01 $mg.kg^{-1}$).

3.2. Biochemical biomarkers

Because the cages were slightly damaged at station A, only 77% of the fish could be sampled (93% for sea bass and 59% for turbot). At station B, 94% of the fish were collected (100% for sea bass and 88% for turbot). No skeletal fish were found in any of the cages, or in the fish gut, from station A or B; we assumed that some fish had escaped when the cage was raised to the surface under difficult meteorological conditions. Therefore, the mortality rate was not assessed in the present study.

Several hepatic biomarkers were analysed, linked to biotransformation of xenobiotics (EROD and GST) and protection against oxidative stress (Catalase) (Fig. 3). Values of EROD activities were found lower in that of sea bass liver compared to turbot. Indeed, EROD activity measured in sea bass sacrificed at the beginning of the experiment (t_0) was below the detection limit, whereas some activities were detected in turbot (0.95 ± 0.22 pmol.min^{-1}.mg^{-1} of protein). For both species, a significant induction of EROD activities was observed in those fish caged in the two stations compared to t_0. This induction was significantly higher in fish from station B (sea bass : 0.90 ± 0.53 pmol.min^{-1}.mg^{-1} protein; turbot : 2.88 ± 0.62 pmol.min^{-1}.mg^{-1} protein) compared to station A (sea bass : 0.40 ± 0.30 pmol.min^{-1}.mg^{-1} protein; turbot : 1.59 ± 0.38 pmol.min^{-1}.mg^{-1} protein) (Anova, $p<0.001$).

For both species, no difference was observed in GST activities between those fish measured from t_0 and those caged in station A. However, GST activities were found significantly higher in fish caged in station B (sea bass : 0.62 ± 0.20 µmol.min^{-1}.mg^{-1} protein; turbot : 0.62 ± 0.14 µmol.min^{-1}.mg^{-1} protein) compared to reference station (A) (sea bass : 0.34 ± 0.16 µmol.min^{-1}.mg^{-1} protein; turbot: 0.44 ± 0.11 µmol.min^{-1}.mg^{-1} protein) (Anova, $p<0.05$).

Values of Catalase activities were found to be about ten times higher in turbot liver compared to sea bass. For CAT activities measured in fish caged in station A, a significant induction was observed in both species (sea bass : 1.40 ± 1.36 µmol.min^{-1}.mg^{-1} protein; turbot : 28.6 ± 7.2 µmol.min^{-1}.mg^{-1} protein) compared to t_0 (sea bass : 1.03 ± 0.22 µmol.min^{-1}.mg^{-1} protein; turbot : 23.1 ± 4.4 µmol.min^{-1}.mg^{-1} protein) (Anova, $p<0.001$). On the contrary, a significant decrease of CAT activities were measured in sea bass caged in station B (0.54 ± 0.18 µmol.min^{-1}.mg^{-1} protein) compared to station A (Anova, $p<0.001$). CAT activities were also lower in turbot from station B (23.1 ± 0.35 µmol.min^{-1}.mg^{-1} protein) compared to station A, but the differences were not significant (Anova, $p=0.149$).

3.3. Relationships with physiological biomarkers

Sea bass caged in station B presented a significant decrease of both specific growth rates, recent growth, Fulton's K condition index, RNA:DNA and TAG:ST ratios, compared to the reference station. For turbot, among all physiological parameters analysed, only the specific growth rates in weight, the Fulton's K condition and the TAG:ST ratio were significantly lower in station B compared to the reference station (Kerambrun et al., submitted for publication). It should be noted that comparisons of growth and condition indices between the

two stations were not the purpose of this study. Consequently, in this paper we have focused only on their relationships with biomarker activities.

The relationships between biomarker activities, biological parameters and sediment chemical contaminants were analysed with a PCA (Fig. 4). The two first axes of the PCA explained 94.26 % of the global inertia in the data with mean explanations for the first axis (74.23 %). The first axis ordinates the stations along a pollution gradient, which appears to be positively correlated with GST activities. On the contrary, the PCA suggests a negative correlation between the contamination gradient and most of the physiological biomarkers. EROD and Catalase activities appear to be more associated with the second axis which seems to differentiate sea bass and turbot among the values of parameters. Among the different biological indicators, the RNA:DNA ratio appears to be the most related with biomarkers activities.

Correlations between biomarkers and physiological parameters (growth and condition indices) were more precisely analysed for each species (Table 2). For each species, by using the data from all individuals collected from the two stations, a Pearson correlation matrix was computed to test the existence of significant links between biochemical and physiological parameters, and to evaluate the strength of these relationships. For sea bass, biochemical biomarkers were well related to physiological biomarkers. CAT activities were significantly and positively correlated with all growth and condition indices. For EROD and GST activities, most correlations were found to be significant and negatively correlated with physiological biomarkers. Among the 12 correlations analysed on sea bass, only 3 were not significant: GST activities with a TAG:ST ratio and EROD with the recent growth (RG) and the Fulton's K index. For turbot, biochemical biomarker activities were slightly related to growth and condition indices, with only significant correlation between EROD activities, the Fulton's K index and the lipid index (TAG:ST), and CAT activities with the lipid index.

4. Discussion

4.1. Biochemical biomarkers responses

Fish, display close physiological relationships to their environment as ectothermic organisms, and as such, are sensitive to environmental disruptions and in particular to chemical stress. As a consequence many authors have begun measuring fish cellular detoxification or defence mechanisms that occur in response to exposure select environmental

xenobiotics (Van der Oost, 2003). The first aim of this study was to analyse the response of three biochemical biomarkers in two marine fish species caged in a harbour polluted area. EROD activities measured in sea bass and turbot were in the same range of values than those measured on the same species by Teles et al. (2004) and Camus et al. (1997) respectively. Ahmad et al. (2009) also found comparable values of GST activities for sea bass, but no reference value of GST activities was found for turbot. These two biotransformation parameters (EROD phase I and GST phase II) activities were found to be higher in sea bass and turbot, caged in the inner part of the harbour, compared to the station located in front of it. Such induction of EROD and GST activity was also recorded in Stien et al. (1998) in sea bass caged in a Mediterranean France harbour and for carp (*Cyprinus carpio*) caged in a Netherlander polluted site (Van der Oost et al., 1998). EROD and GST are known to be induced by several organic contaminants such as PAHs and PCBs (Deviller et al., 2005; Castro et al., 2004 ; Fragoso et al., 2006). As higher levels of PAHs were detected in station B compared to station A, the increase of these biotransformation enzymes could be explained by these organic compounds. However, other organic compounds not measured in this study such as dioxin could have enhanced these enzymes. Indeed, an EROD induction was observed in juvenile sea bream, *Sparus aurata*, exposed to a dioxin mixture (Abalos et al., 2008).

Complementary to these two biotransformation parameters, the catalase was measured to analyse the antioxidant status of juvenile fish. Catalase is an important enzyme in antioxidant defence system protecting animals from oxidative stress. Induction of biotransformation metabolism produces highly reactive oxygen species (ROS) as by-products leading to oxidative stress (Jifa et al., 2006). In this way, the antioxidant defence system may be induced in cells as response to ROS. In our study, a decrease of catalase activities was observed for both juvenile sea bass and turbot, even though the decrease was not significant for turbot. This inhibition was also observed in sea bass exposed to cadmium (Romeo et al., 2000) and pacu (*Piaractus mesopotamicus*) exposed to copper (Sampaio et al., 2008). Palace et al. (1992) estimated that possible mechanisms by which metal produces lower catalase activity, may include direct metal-mediated structural alteration of the enzyme and depression of catalase synthesis. This phenomenon, could explain the decrease of catalase activities which occurred in our study, since sediment from station B showed concentrations of Cd, Hg and Zn higher than ERL guidelines.

In complex chemical mixtures, such as those found in the polluted sediments of the studied harbour, any biomarkers regulation was likely to be the net result of additive, synergistic or antagonistic chemical interactions. Indeed, while many studies have shown the

induction of biotransformation enzymes by organics compounds, several studies have shown an inhibition of EROD activities by metals. EROD decrease was observed for sea bass, *Dicentrarchus labrax* (Viarengo et al., 1997) and leaping mullet, *Liza saliens* (Bozcaarmutlu and Arinç, 2004) following metal exposure. This inhibition was explained by binding of metal ions to the sulfhdryl group of the enzymes or generation of reactive oxygen species (ROS) (Bozcaarmutlu & Arinç, 2004). On the other side, recent studies have shown in vivo GST induction in zebra fish, *Brachydanio rerio*, exposed to heavy metals (Paris-Palacios et al., 2000) and a positive correlation was found between copper and GST induction in grey gold mullet, *Liza aurata* (Pereira et al., 2009). In the same way, various responses of CAT activity have been observed in fish exposed to organic or metallic contaminants in both field and laboratory experiments. Indeed, CAT has been shown to be either induced or inhibited by metals depending on the dose, the species or the route of exposure (Romeo et al., 2000; Sanchez et al., 2005).

4.2. Comparison between biochemical and physiological biomarkers

In the last years, the use of biochemical biomarker in fish has been enhanced by increased information about influencing factors (Kammann et al., 2004). However, the relationships between molecular responses and effects at higher levels of biological organisation have not been examined in great detail. In the second part of this study, we have compared the biochemical biomarkers with several growth and condition indices measured from the same fish. Examination of individual fish growth and condition is one method that has been successfully used to compare habitat quality among different juvenile nursery areas (e.g. Sogard, 1992; Meng et al., 2000, Gilliers et al, 2006, Searcy et al., 2007; Amara et al., 2007). The use of growth and condition as indices of habitat quality is based on the assumption that larger, faster-growing fish are healthier and hence experience more favourable environmental conditions than smaller, slower-growing fish. In a previous study, we analysed the relationships between chemical contamination and biological performance of juvenile marine fish using the caging method (Kerambrun et al., submitted for publication). We found a general decrease in growth and condition indices of juvenile fish caged in the contaminated station (B).

4.2.1. Sea bass

The results of comparison of parameter's means between the two stations, show that the inductions of EROD and GST, and the decrease of CAT activities found on sea bass caged in station B, are related to a significant decrease of both specific growth rates, recent growth, Fulton's K condition index, RNA:DNA and TAG:ST ratios. Using multiple correlations performed with the data from all the fish collected from both stations, no significant correlation was observed between EROD activities and the otolith's recent growth, or the Fulton's K index. This latter morphometric index is commonly used as indicators for an individual's general well-being, which represents a more longer-term indicator compared to some biochemical indices like RNA:DNA ratio (Vasconcelos et al., 2009). Indeed, Ferron (2000) suggested that morphological indicators have lower sensitivity, longer latency and slower dynamics than nucleic acids and lipids which respond on a shorter scale. The same results were obtained for juvenile sole exposed for 15-days to copper (Fonseca et al., 2009). Biochemical biomarkers response represents short-term responses to contaminant and so they would be less related to longer-term physiological biomarkers. However, GST activities were significantly correlated with recent growth and the Fulton's condition index suggesting the potential higher relevance of this biotransformation enzyme than EROD.

High levels of both EROD and GST activities were significantly correlated with reductions of the somatic growth rates, RNA:DNA ratios and lipid index of sea bass caged in the contaminated station. This relation suggests that there are metabolic costs associated with the synthesis of these proteins or with detoxification processes (Rose et al., 2006). Indeed, as growth is an integration of many processes (Morales-Nin et al., 2007), such changes in fish metabolism could result, at a higher level, to growth impairments. Previous studies have analysed biochemical biomarkers and growth measurement relationships. Cao et al. (2010) reported that after cadmium exposure, the decrease in the growth of juvenile Japanese flounder (*Paralichthys olivaceus*), was related to inhibition of CAT and GST activities. The growth reduction of the greenback flounder (*Rombosolea tapirina*) exposed to contaminated sediment was also related to an induction of EROD activities (Mondon et al., 2001). On the contrary, in Abalos et al. (2008), the increase of EROD and GST activities in juvenile gilthead seabream (*Sparus aurata*) was not related to growth which showed no difference between dioxin-exposed and non-exposed fish.

The decrease of catalase activities measured in sea bass was associated with a decrease of all physiological biomarkers. Indeed, catalase activities showed the strongest correlations with growth and condition indices compared to the two biotransformation parameters measured. Bagnyukova et al. (2005) showed that a catalase inhibition in goldfish (*Carassius auratus*)

was associated with lipid oxidative damage products due to an oxidative stress from the absence of cell protection by this antioxidant enzyme. They also showed that the response to catalase depletion involves compensatory changes in the activities of other antioxidant enzymes. This change in cell metabolism in response to the decrease of catalase activities could have played a role in the decrease of sea bass physiological status observed in station B.

4.2.2. Turbot

Despite high levels of biotransformation parameters found in turbot caged in station B, no difference in growth in length and RNA:DNA ratios was observed. These low responses of turbot growth parameters have been explained in our previous results by the non optimal environmental parameters for juvenile turbot growth (sediment grain size, turbidity and feeding behaviour) as well as for reference station (A). Cages are a valuable tool for comparing the relative growth of small juvenile fish in different habitats but are also known to have certain limitations (Able et al., 1999; Phelan et al., 2000; Oikari, 2006). Cages confine fish to a small space, preventing movements to surrounding areas i.e. for food searching. The analysis of stomach of the two species indicated no food limitation as also observed in other caging studies (Sogard, 1992; Kamermans et al., 1995). Energetic studies have shown that the metabolic rates of flatfish can increase significantly when they are forced to bury in sediments with suboptimal grain sizes, or do not have access to sediments at all (Howell and Canario, 1987). The non optimal grain size of the sediment, or the fact that the cage infrastructure may have limited the turbot burying, could explain the decrease of turbot condition in station A even if the sediment was slightly contaminated. Consequently, among the different physiological parameters analysed in turbot, only the specific growth rate in weight, the Fulton's K condition index and the lipid index could be related with the significant EROD and GST induction. In particular, using multiple correlations performed with the data from all fish collected in both stations, the TAG:ST ratios were the ones significantly correlated with both EROD and CAT activities. Lipid depletion has been identified as a general metabolic response to stress (Claireaux et al., 2004). TAGs are the principal reserve of energy in teleosts and the first components to be mobilised during periods of stress (Galois et al., 1990). Hakanson (1989) reported that TAGs were used preferentially for short-term energy need. Their decrease could be the direct consequence of the xenobiotic detoxification and regulation which involve both passive and active mechanisms and therefore require energy (Alquezar et al., 2006). Previous studies have reported similar depletion in lipid reserves of fish subjected to metal trace exposure (Rowe, 2003; Fonseca et al., 2009).

Since turbot growth appears to have been influenced by other environmental factors with additional chemical contaminant effects, the relationships between biochemical biomarkers and growth parameters could not be established for this species. These differences in behaviour between a demersal fish like the sea bass and a benthic fish like the turbot, could explain the variations observed in our study to fish responses to chemical contamination. These results show the importance in the choice of the fish species suitable for caging studies in order to minimise physiological stress in caged fish. Because of species specific differences in behaviour, feeding activities and physiological processes, fish biomarker responses and physiological parameters sensitivity, must be analysed carefully.

Conclusions

This multibiomarker approach allowed us to assess different levels of fish responses to chemical contamination. This study was a first approach of simultaneous comparison of both biochemical and physiological biomarkers in a caging study. For individual sea bass, biochemical biomarkers, growth and condition indices such as the Fulton's K condition index, the RNA:DNA ratio and the lipid storage index, showed numerous significant correlations. Conversely, there were only a few significant correlations for turbot, suggesting a species-specific response. However, even if biochemical biomarkers responses and fitness are a direct measure of an individual fish's stress and health, they are not specific to fish stress responses to pollutants, as several other biotic and abiotic stressors can affect them. Environmental conditions must be considered when one is integrating both biochemical and physiological biomarkers responses with pollution stress. This can explain the species-specific response we observed.

Although based on the comparison of only two stations, our data support the use of caged juvenile sea bass as sentinel species due to some original ecological features of this species (*e.g.* large distribution, eurythermal and euryhaline species). We found that the combined use of several biochemical biomarkers and physiological responses such as growth and condition indices can improve fish health determination, and may provide a useful tool to monitor and assess habitat quality for juvenile fishes.

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Figure legends

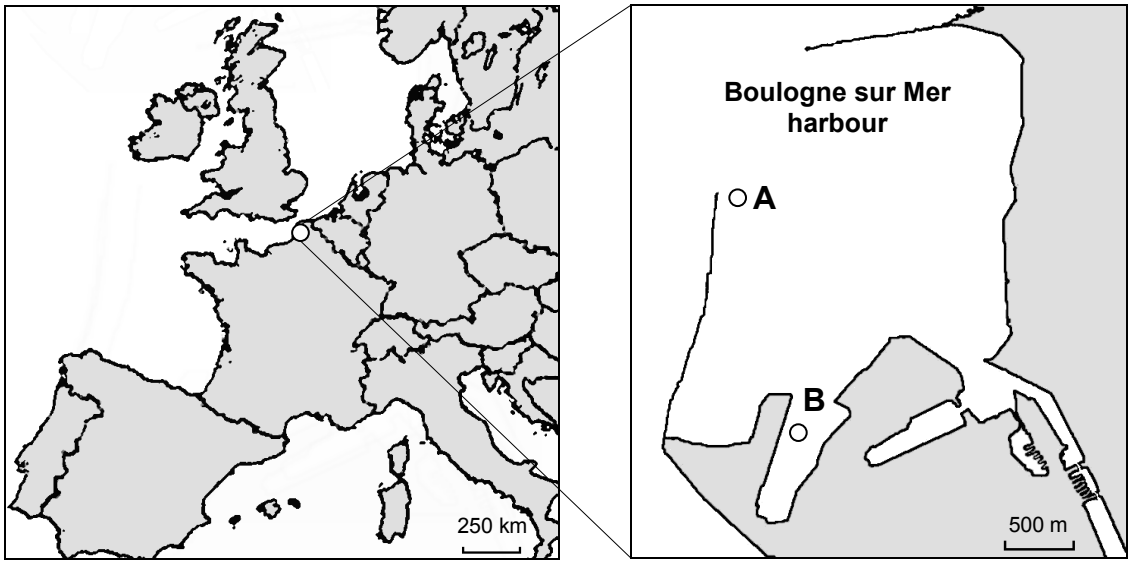
Fig. 1. Locations of the two caging stations at the harbour of Boulogne sur Mer, France.

Fig. 2. Mean (± SD) sediment metal concentrations ($mg.kg^{-1}$ dry weight) in the two caging stations (▩ A and ■ B). * Concentration that exceeds the ERL (effects range-low) defined by Long et al. (1995).

Fig. 3. Differences in Ethoxyresorufin-O-deethylase (EROD), Glutathione S-transferase (GST) and Catalase (CAT) activities of juvenile sea bass and turbot caged in station ▩ A and ■ B (□ t_0 : reference). Data represent mean (± SD) biochemical biomarkers activities and for each species, (^{1}) represent significant difference ($p<0.05$) compared to "t_0" and (^{2}) compared to "station A".

Sea bass : n = 15 (t_0), n = 14 (A), n = 18 (B); Turbot : n = 15 (t_0), n = 10 (A), n = 14 (B).

Fig. 4. PCA based on biomarker responses (Ethoxyresorufin-O-deethylase -EROD- , Glutathione S-transferase -GST- and Catalase -CAT) of juvenile sea bass (□) and turbot (●) in relation with physiological biomarkers (specific growth rate in length -GL- and weight -GW-, recent growth -RG-, Fulton's condition index -K-, RNA:DNA and Triacylglycerol on sterol -TAG:ST- ratios) and chemical contamination (metals and total PAHs) measured in caging station A and B.


250 km
Boulogne sur Mer
harbour
A
B
500 m

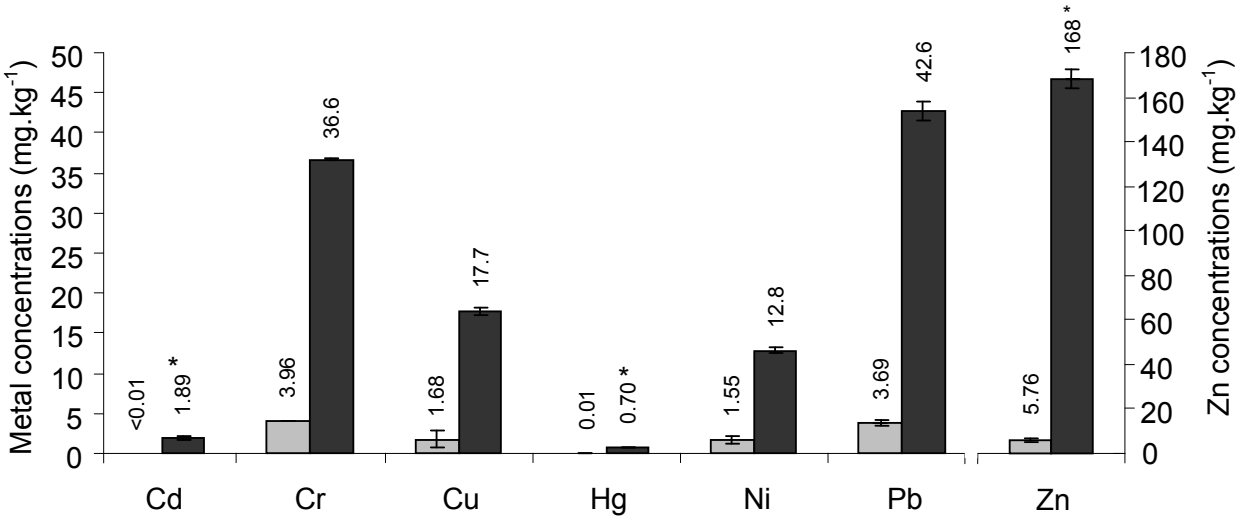

Metal concentrations (mg.kg-1)
Zn concentrations (mg.kg-1)
50
45
40
35
30
25
20
15
10
5
0
180
160
140
120
100
80
60
40
20
0
<0.01
1.89 *
3.96
36.6
1.68
17.7
0.01
0.70 *
1.55
12.8
3.69
42.6
5.76
168 *
Cd
Cr
Cu
Hg
Ni
Pb
Zn

Fig. 2

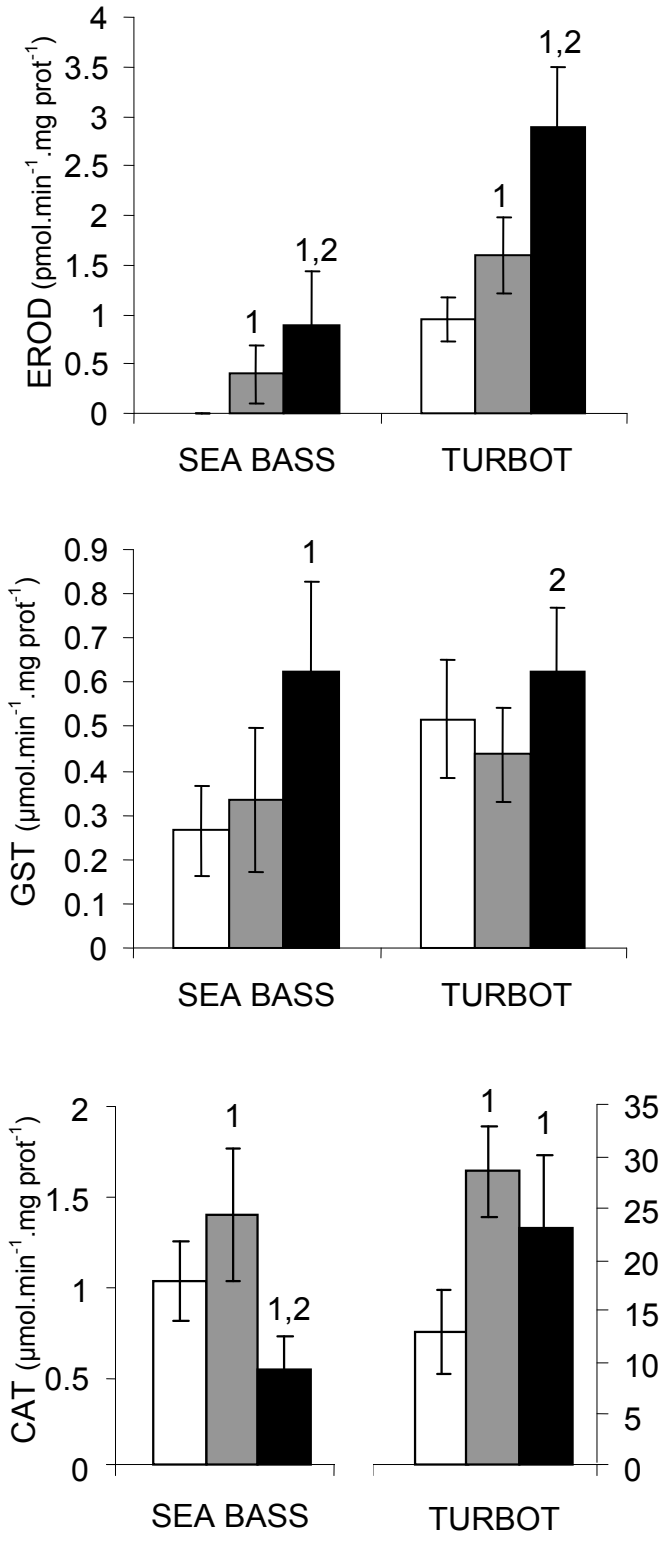

EROD (pmol.min-1.mg prot-1)
4
3.5
3
2.5
2
1.5
1
0.5
0
1
1,2
1
1,2
SEA BASS
TURBOT
GST (µmol.min-1.mg prot-1)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
SEA BASS
TURBOT
CAT (µmol.min-1.mg prot-1)
2
1.5
1
0.5
0
1
1,2
1
1
35
30
25
20
15
10
5
0
SEA BASS
TURBOT

Fig. 3

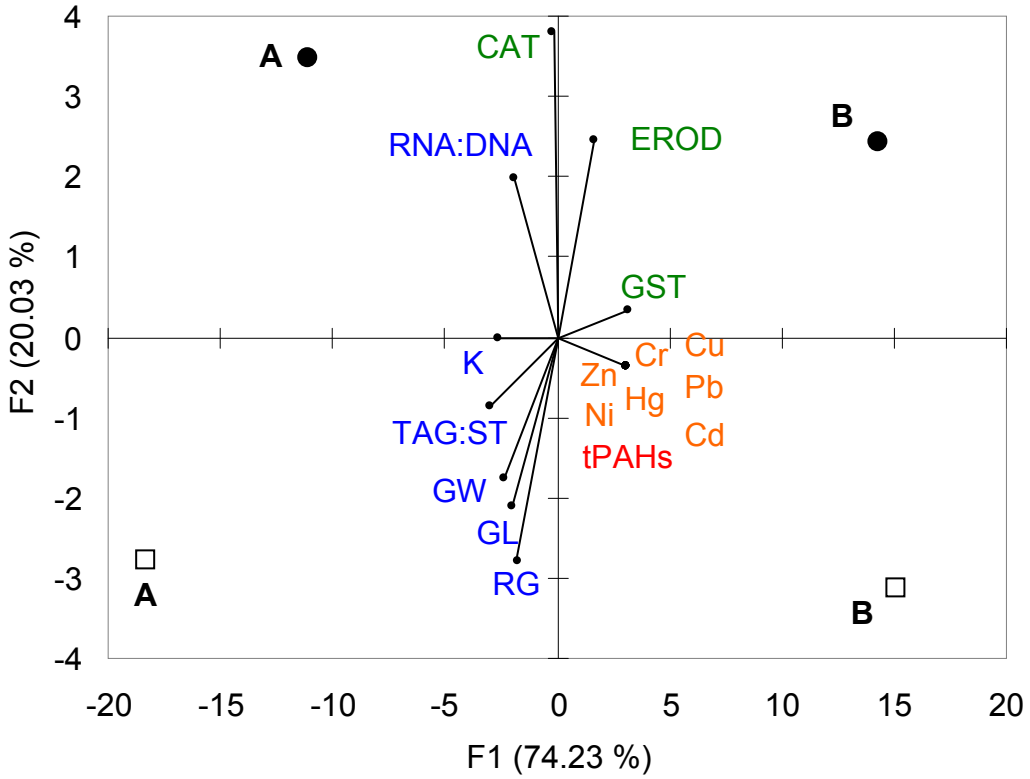

A
B
CAT
EROD
RNA:DNA
GST
K
Cr
Cu
Zn
Pb
Hg
Ni
Cd
TAG:ST
tPAHs
GW
GL
RG
A
B
F2 (20.03 %)
F1 (74.23 %)
4
3
2
1
0
-1
-2
-3
-4
-20
-15
-10
-5
0
5
10
15
20

Fig. 4

Table legends

Table 1

Mean (± SD) PAH and PCB concentrations ($mg.kg^{-1}$ dry weight) in sediment from the two caging stations (A and B).

Table 2

Pearson's correlations between biochemical biomarker responses (Ethoxyresorufin-O-deethylase -EROD- , Glutathione S-transferase -GST- and Catalase -CAT) of juvenile sea bass (□) and turbot (●) in relation with physiological biomarkers (specific growth rate in length -GL- and weight -GW-, recent growth -RG-, Fulton's condition index -K-, RNA:DNA and Triacylglycerol on sterol -TAG:ST- ratios) measured in juvenile sea bass (a) and turbot (b) caged in stations A and B. Significant correlation for $p^* < 0.05$, $p^{**} < 0.01$ and $p^{***} < 0.001$. Sea bass : n = 32 ; Turbot : n = 24

Table 1

	A	B
PAHs		
Naphtalene	<0.05	<0.05
Acenaphtylene	<0.05	<0.05
Acenaphtene	<0.05	<0.05
Fluorene	<0.05	<0.05
Dibenzo(a.h)anthracene	<0.05	<0.05
Phenanthrene	<0.05	0.09 ± 0.05
Anthracene	<0.05	<0.05
Fluoranthene	<0.05	0.10 ± 0.05
Pyrene	<0.05	0.08 ± 0.05
Benzo(a)anthracene	<0.05	<0.05
Chrysene	<0.05	0.06 ± 0.05
Benzo(b)fluoranthene	<0.05	0.08 ± 0.05
Benzo(k)fluoranthene	<0.05	<0.05
Benzo(a)pyrene	<0.05	0.05 ± 0.05
Benzo(ghi)perylene	<0.05	<0.05
Indeno(123-cd)pyrene	<0.05	<0.05
Total PAHs		0.46 ± 0.07
PCBs		
Total PCBs (congeners 28 – 52 – 101 – 118 – 138 – 153 – 180)	<0.01	<0.01

Table 2

	GW	GL	RG	K	RNA:DNA	TAG:ST	EROD	GST
EROD	-0.41*	-0.49*	-0.25	-0.22	-0.43*	-0.45*	-	
GST	-0.57**	-0.56***	-0.42*	-0.53**	-0.48*	-0.37	0.40*	-
CAT	0.74***	0.70***	0.50**	0.60***	0.47*	0.63***	-0.30	-0.59***

a.

	GW	GL	RG	K	RNA:DNA	TAG:ST	EROD	GST
EROD	-0.45	-0.04	0.34	-0.46*	0.04	-0.47*	-	
GST	-0.06	0.15	0.15	-0.14	0.19	-0.24	0.27	-
CAT	0.01	-0.20	0.01	0.33	0.08	0.62**	-0.50*	-0.25

b.